The University of Chicago

SIZE OF EXPLANT AND VOLUME OF MEDIUM IN TISSUE CULTURES

A DISSERTATION SUBMITTED TO THE
GRADUATE FACULTY IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY

By
RALPH MORRIS BUCHSBAUM

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SIZE OF EXPLANT AND VOLUME OF MEDIUM IN TISSUE CULTURES

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FOUR FIGURES

INTRODUCTION

Most of the results of experiments and observations on the relation between the concentration of animals and their physiological processes show that, within limits, the rates of metabolism, growth, and reproduction are roughly inversely proportional to the concentration of the animals. Yet there is some important evidence indicating that there is an optimum concentration of animals, greater than the minimum, at which they use most oxygen, grow and reproduce most rapidly, or survive longest under favorable or more particularly under unfavorable conditions.

Harmful results of crowding have been described for bacterial cultures by many workers. These harmful effects have been claimed by some to be due not so much to exhaustion of the food supply, but to the accumulation of some toxic substances, such as carbon dioxide. However, others believe that the slowing down of crowded bacterial cultures is due to nutritional effects. Buchanan ('28) states that the rate of growth of bacteria is proportional to the concentration of food materials during the early phases of growth, but conditions become progressively more unfavorable to growth, due to decrease in concentration of nutrients and to the accumulation of toxic substances.

Woodruff ('11, '14) found that the number of Paramecium in a culture is limited by the accumulation of toxic substances

and the decrease in the amount of their food. He placed P. aurelia in varying volumes of culture medium. The larger volumes showed the greatest rate of reproduction. When the medium was changed every day, the animals showed an increase in the rate of reproduction. He concluded that the greater the volume, the more rapid is the rate of division.

On the other hand, bacteriologists find that, within certain limits, smaller inocula have longer lag phases than larger inocula. Similar effects have been found for yeast cultures (Buchanan, '28). Larger inocula have greater survival value when transferred to toxic media, as in the case of Churchman and Kahn's experiments ('21), in which five to fifteen organisms (B. coli) did not grow on gentian violet agar, though transplants of thirty grew regularly. Robertson ('23, '24) found that a ciliate Enchelys reproduced from two and a half to ten times as rapidly when two individuals were placed together in a drop of culture medium, as compared with one individual in the same volume of medium. Robertson ('24) called this phenomenon 'allelocatalysis.' He defined it as "the acceleration of multiplication by the contiguity of a second organism in a restricted volume of nutrient medium." Greenleaf ('24), on Paramecium and Pleurotricha, and Peskett ('24), on yeast cultures, failed to find the phenomenon. Cutler and Crump ('25) repeated the observations, using Colpidium, a ciliate, and found no allelocatalytic effect. Calkins ('26) placed one, two, three, and four Uroleptis into fresh medium. The rate of division was inversely proportional to the initial number inoculated. Meyers ('27) found that "increasing the numbers of individuals (of Paramecium) present in a given volume does not increase the rate of reproduction." Yocom ('28) claimed that Oxytricha, a ciliate, in the smaller volumes showed greater division rates than those isolated into larger volumes. Petersen ('29) did not find allelocotalysis in a series of experiments using the small volumes used by Cutler and Crump, and Myers, but in relatively larger volumes in a second series of experiments, she found an increase in rate of division per animal following the introduction of a second Paramecium.

There is a considerable amount of other data, some of which supports while the rest denies the allelocatalytic phenomenon. Allee ('31) gives a detailed account of the literature in this controversy. In regard to Robertson's allelocatalysis, Allee says:

> The evidence demonstrates that reproductive rate does not always depend on the number of the original transplants; that, in fact, under many conditions and with many organisms it has not yet been shown to have any positive correlation. All are agreed that in the end the rate of reproduction falls sooner in small subcultures seeded with more than one organism and more rapidly than it does when a single individual is isolated. However, the repeated experience of Robertson, supported by Yocum's work in so far as it covers the same ground, and by the work of Petersen with larger volumes of culture medium, demonstrates that true acceleration of the rate of reproduction may occur associated with the introduction of more than one organism into a limited amount of medium.

It was accordingly thought of interest to study the relation in tissue culture between the size of fragments of tissue and the volume of the medium, to find if possible whether there is any effect comparable to the allelocatalytic phenomenon as observed by the workers mentioned above. In other words, whether there can be discovered any beneficial effect of crowding of cells into a limited volume of medium. The specific problem of this paper can be stated diagrammatically as follows: Drops of medium are placed on four cover slips, two, (*a*) and (*b*), having (say) twice the volume of medium of the other two, (*c*) and (*d*), the size of explant of (*a*) and (*c*) being then twice that of (*b*) and (*d*).

In view of the work of Woodruff, Cutler and Crump, Peskett, and others, the smaller piece in the larger volume of medium would be expected to grow more than the larger piece in the smaller volume, or $b > d \geqq a > c$. The explanation given is that the greatest amount of food including oxygen is present in the larger volumes of medium and the concentration of metabolic products would be smaller. According to the allelocatalytic hypothesis, the opposite relation would be expected: the larger piece of tissue in the

smaller volume is expected to grow more than the smaller piece of tissue in the larger volume, or $c > a \geqq d > b$, in respect to growth. The explanation for this may be that the larger pieces of tissue contain greater numbers of cells, which supposedly can condition the medium to their more favorable growth, more than can the smaller fragments of tissue.

MATERIAL AND METHODS

Tissue for culturing was obtained aseptically from the heart of six-day embryonic chicks, or from the periosteum of the femur of twelve-day embryonic chicks. Pieces, 3 or 4 mm.^{3} in volume, were transferred to cover slips upon which had been placed heparinized chicken plasma[1] and chick-embryo extract.[2].

After the medium had clotted, the cover slip was inverted over a vaselined depression slide, sealed with paraffin and placed in the incubator at 39°C. After forty-eight hours, the cultures showed a ring of more or less radially arranged cells, chiefly spindle cells, macrophages, and blood cells. The cover slip was removed, placed culture-side up on the table, and the tissue excised with four cuts of a sharp knife. The cuts were made just inside the periphery of growth. The excised fragment was placed in fresh medium, or first divided and the pieces placed in separate drops of medium. After about ten passages, most of the cells appeared morphologically homogeneous, with a few macrophage-like cells persisting.

The growth of two individual cultures derived from different sources may vary considerably. As Ebeling ('21) has shown, cells in as nearly the same physiological condition as possible should be used, namely, halves of a single parent culture. One-half is used as the control culture of the other

[1] Twenty cubic centimeters of blood were drawn from the heart of a chicken into a syringe containing 1 cc. of a 1:2500 'purified' heparin solution in 0.85 per cent NaCl solution. After centrifuging, the plasma was drawn off and kept in ampules in the refrigerator.

[2] Seven-day-old chick embryos were minced with scissors. The pulp mixed with an equal volume of Tyrode solution was centrifuged. The supernatant fluid was ampulled and stored in the refrigerator until used. Fresh extract was made weekly.

which is experimented upon. A symmetrical culture was divided into two pieces, and these were transferred to separate drops of medium. The method described by Ebeling ('21) was used in studying relative differences in growth of tissues when their initial relative size and volume of medium was varied. The explanted tissues were projected, enlarged, upon a sheet of paper and the outlines drawn. After forty-eight hours' growth, the cultures were again projected and the new outlines drawn. The areas within the outlines were measured with a planimeter and the figures computed to square millimeters of actual area, the magnification of the optical system being known. The expression of areas in terms of standard dimensions is highly desirable for purposes of comparison, but, unfortunately, much of the work published is in terms of various arbitrary units. As Ebeling ('21) and Mayer ('30) have shown, the area of the culture is large compared with its thickness; the growth of a carefully made culture in a widely spread thin drop of plasma lies in a relatively thin layer. Hence the area may be taken as an approximate measure of growth. The area after forty-eight hours minus the original area is the absolute increase, and is composed of cells which have multiplied and migrated away from the central explant. The area of absolute increase divided by the area of the originally explanted fragment is the relative increase of growth. The differences in the relative increases of the pairs of cultures are examined statistically as described below.

EXPERIMENTAL RESULTS

Nine series of experiments are here reported, in which the volume of the culture medium and the relative size of the explants were varied. The results of the experiments are summarized in table 7.

In the first series of experiments the two fragments were divided as equally as possible and placed in equal volumes (four drops; 1 drop = 0.01 cc.) of culture medium. Twenty pairs of cultures were made, in this as well as in the other

series reported. Cultures were projected soon after explanting and also after forty-eight hours, and the outlines of the cultures as projected were drawn as described above. The areas were measured, the relative increases computed, and the data as presented in table 1 were examined statistically by 'Student's ('25) method. The difference between the relative growths was 2.3 per cent. The probability was 0.6618. This means that there is roughly an even chance of obtaining the same result by a random distribution, and therefore the average ratio of the areas of the original explants of 0.89 did not show a significant difference in relative growth of the two fragments.

In the second series of experiments, the ratio of the two explanted fragments of tissue was 0.58. The pieces were explanted into equal volumes of medium (two drops). Here the smaller fragment produced on the average 33.8 per cent more growth than the larger fragment, with a probability of 0.0160. This means that there are only sixteen chances in 1000 of obtaining the same result by a random distribution. See table 7, (2).

In the third series of experiments (table 7 (3) and table 2) the ratio of the sizes of the original fragments was 0.71. Here the volume of medium was double that of series (2) namely, four drops. The smaller fragment grew significantly more in the forty-eight-hour period than the larger by 29.5 per cent The probability of significance was 0.0000, or less than one chance in 10,000 of obtaining the same distribution at random.

It was suspected that the method of dividing the parent culture (fig. 1) in series (2) and (3) may have given a disproportional amount of the denser central portion of the culture to the larger fragment such that the results may not be strictly comparable. To check this (series 4), the tissue was cut radially as shown in figure 1, such that the two fragments consisted of proportionate parts of the denser and thinner areas of the culture. The ratio of the two parts was 0.55. The volume of the medium was the same as in series (3). The smaller fragment grew more than the larger by 40.5 per cent, giving a probability of 0.0034.

Table 1

Relative Growth of Two Approximately Equal Fragments of a Culture of Spindle Cells

No.	Area of smaller explant mm.²	Relative increase	Ratio of areas of explants	Area of larger explant mm.²	Relative increase	Difference in relative increase
1.	1.60	2.59	0.94	1.71	2.80	-0.21
2.	.38	4.66	.83	.46	5.92	-1.26
3.	..59	2.36	.89	.66	3.18	-0.82
4.	.40	10.00	.91	.44	9.53	0.47
5.	.54	11.96	.97	.55	10.82	1.14
6.	.71	5.92	.97	.73	9.62	-3.70
7.	.52	9.62	.96	.54	10.05	-0.43
8.	.55	19.90	.90	.61	14.57	5.33
9.	1.50	5.05	.85	1.77	5.38	-0.33
10.	1.12	3.87	.91	1.24	4.64	-0.77
11.	.97	6.63	.87	1.12	5.74	0.89
12.	.88	5.82	.79	1.12	7.45	-1.63
13.	1.12	6.90	.77	1.46	5.50	1.40
14.	1.31	9.90	.79	1.65	6.15	3.75
15.	1.13	8.12	.93	1.22	8.94	-0.82
16.	1.55	4.77	.91	1.70	6.05	-1.28
17.	.64	10.15	.94	.68	10.38	-0.23
18.	.59	22.55	.92	.64	21.26	1.29
19.	1.11	8.67	.83	1.34	8.76	-0.09
20.	1.23	11.00	.92	1.34	9.87	1.13
Mean	0.922±0.060	8.522±0.780	0.890±0.090	1.049±0.070	8.330±0.609	+0.192±0.291

Table 2

Relative Growth of Small and Large Fragments of Tissue of Spindle Cells

No.	Area of small explant mm.^{2}	Relative increase	Ratio of areas of explants	Area of large explant mm.^{2}	Relative increase	Difference in relative increase
1.	0.47	7.14	0.74	0.64	3.87	3.27
2.	.34	7.03	.56	.61	4.74	2.29
3.	.81	8.98	.69	1.17	7.38	1.60
4.	.33	19.62	.65	.51	14.25	5.37
5.	.40	18.25	.63	.63	9.57	8.68
6.	.51	3.78	.65	.79	4.66	-0.88
7.	.49	15.70	.83	.59	12.20	3.50
8.	.57	9.60	.79	.72	6.70	2.90
9.	.57	14.54	.89	.64	16.02	-1.48
10.	.47	12.28	.74	.64	7.09	5.19
11.	.56	17.82	.77	.73	14.55	3.27
12.	.48	13.42	.58	.83	8.36	5.06
13.	.65	15.02	.84	.77	11.22	3.80
14.	.37	16.25	.90	.41	11.73	4.52
15.	.43	19.00	.71	.61	10.26	8.74
16.	.41	14.82	.48	.85	8.78	6.04
17.	.72	8.27	.78	.92	7.41	0.86
18.	.61	16.03	.77	.79	9.28	6.75
19.	.47	13.12	.76	.62	10.85	2.27
20.	.13	16.48	.47	.28	9.38	7.10
Mean	0.489±0.016	13.358±0.647	0.712±0.019	0.688±0.028	9.415±0.504	+3.943±0.421

If the ratio of the size of the two explants is still further decreased to 0.19 as in series (5), table 7, the difference in growth is still more striking. The smaller fragment grew better than the larger fragment by 82.8 per cent, with a probability of 0.0016.

In the sixth series of experiments the two fragments were nearly equal in size, 1.07 ratio. They were placed in two and four drops, respectively. The fragment in the larger volume grew more rapidly than that in the smaller volume by 24.9 per cent (probability 0.0088). If these data are rearranged (series 6′, table 7) with respect to size irrespective of volume of medium, the ratio of the areas of the original ex-

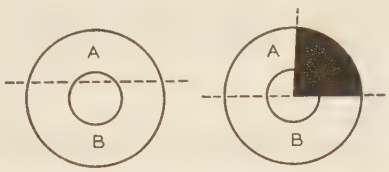

Fig. 1 Showing methods of dividing cultures. The inner circles represent densed portions of the parent cultures, remnants of the previous explant, the outer circles represent the new growth. The dotted lines indicate lines of cutting. The shaded portion was discarded. *a* and *b*, fragments explanted. See table 7. Series 1, 2, 3, 6, 9 on the left. Series 4, 5, 8 on the right.

plants is 0.76. The smaller fragment grew more than the larger by 18.3 per cent, with a probability of 0.078, which is indicative, but not formally significant. Thus it can be seen that both area and volume are factors that influence the growth in these cultures.

In series (7), shown in table 3, one of the fragments was placed in four drops of medium, the other nearly equal in area (ratio 1.02) was placed in twelve drops. Here the same result was obtained, namely, the greater growth was seen in the greater volume. The difference was 38.5 per cent (probability 0.0022). If these data are tabulated according to size of fragment (series 7′, table 4), the ratio of the sizes is seen to be 0.74, and the smaller fragment grew better than the larger by 31.7 per cent, with a significant probability of 0.0218.

Table 3

Relative Growth of Two Approximately Equal Fragments of Tissue in 12 and 4 Drops of Medium

No.	Area of explant in 12 drops. mm.^{2}	Relative increase	Ratio of areas of explants	Area of explant in 4 drops. mm.^{2}	Relative increase	Difference in relative increase
1.	0.52	25.42	0.63	0.83	8.45	16.97
2.	74	15.10	1.07	.69	14.20	.90
3.	.87	13.16	1 16	.75	15.98	- 2.82
4.	.49	22.39	.49	1.00	5.17	17.22
5.	.44	14.84	.61	.72	2.34	12.50
6.	.53	9.28	.82	.61	1.21	8.07
7.	.47	18.65	.80	.59	12.34	6.31
8.	.34	20.08	.54	.63	3.59	16.49
9.	.70	14.15	1.75	.40	12.33	1.82
10.	.53	15.97	.78	.68	4.53	11.44
11.	.45	14.29	1.29	.35	3.66	10.63
12.	.62	7.32	1.35	.46	12.21	−4.89
13.	.68	8.39	1.15	.59	8.28	.11
14.	.74	21.03	.81	.91	10.51	10.52
15.	.95	14.30	1.17	.81	7.40	6.90
16.	.44	14.43	.76	.58	9.78	4.65
17.	.49	7.40	.88	.56	7.66	− .26
18.	.84	10.90	1.65	.51	8.45	2.45
19.	.62	11.00	1.82	.34	14.33	−3.33
20.	.27	15.05	.93	.29	18.05	−3.00
Mean	0.587±0.027	14.658±0.766	1.023±0.059	0.615±0.029	9.024±0692	+5.634±1.072

Table 4

Relative Growth of the Fragments of Tissue of Table III Arranged According to Size (Neglecting Volume of Medium) Cultures in 12 drops of medium indicated by "*"; others in 4 drops.

No.	Area of small explant mm.²	Relative increase	Ratio of areas of explants	Area of large explant mm.²	Relative increase	Difference in relative increase
1.	0.52 *	25.42	0.63	0.83	8.45	16.97
2.	.69	14.20	.93	.74 *	15.10	- .90
3.	.75	15.98	.86	.87 *	13.16	2.82
4.	.49 *	22.39	.49	1.00	5.17	17.22
5.	.44 *	14.84	.61	.72	2.34	12.50
6.	.53 *	9.28	.82	.61	1.21	8.07
7.	.47 *	18.65	.80	59	12.34	6.31
8.	.34 *	20.08	.54	.63	3.59	16.49
9.	.40	12.33	.57	.70 *	14.15	- 1.82
10.	.53 *	15.97	.78	.68	4.53	11.44
11.	.35	3.66	.77	.45 *	14.29	-10.63
12.	.46	12.21	.74	.62 *	7.32	4.89
13.	.59	8.28	.87	.68 *	8.39	- .11
14.	.74 *	21.03	.81	.91	10.51	10.52
15.	.81	7.40	.85	.95 *	14.30	- 6.90
16.	.44 *	14.43	.76	.58	9.78	4.65
17.	.49 *	7.40	.88	.56	7.66	- .26
18.	.51	8.45	.61	.84 *	10.90	- 2.45
19.	.34	14.33	.55	.62 *	11.00	3.33
20.	.27 *	15.05	.93	.29	18.05	- 3.00
Mean	0.508±0.022	14.069±0.851	0.740±0.020	0.694±0.026	9.612±0.708	+4.457±1.202

The action of the two factors: size of explant and volume of medium is clearly shown.

Series (8), table 6, represents data obtained by dividing a culture into four fragments. Two of these were placed close together in the same medium; the other two were planted about 1 cm. apart in another four drops of medium. The results showed 11.9 per cent larger total growth in favor of the separated pieces. However, the probability was 0.1234, which means that there is only a slight indication that the larger total growth of the separated pieces is significant. These same data were tabulated to compare the relative growth of the two separated pieces of tissue in the same medium. See series (8′), table 5. The average ratio of the areas of the two pieces was 0.64. The smaller fragment grew more than the larger by 28.3 per cent. The probability was 0.0100, the difference is thus significant.

Another possible combination was to grow the smaller fragment in the small volume and the larger fragment in the larger volume (series 9), table 7. The smaller fragment placed in four drops averaged 0.56 of the larger fragment placed in eight drops of medium in area of explant. The smaller fragment grew 34.8 per cent more than the larger fragment despite the smaller volume; the probability was 0.0008. This probability means that the effect of size of explant is greater than that of volume of medium.

DISCUSSION

The results of the experiments here reported clearly show that under the conditions of the experiments, namely, using volumes of two to twelve drops (0.02 to 0.12 cc.), and cultures of embryonic-chick spindle cells, the smaller of two fragments grows more than the larger, within the limits of ordinary hanging-drop cultures (0.1 to 2.5 mm.^{2} as the area of explants). It appears from a study of the experiments in which varying volumes were used (series 6, 7, table 3, and especially from series 8′, table 5, in which the two fragments were placed in the same drop of medium) that the size of explant is a

Table 5

Relative Growth of Small and Large Fragments of Tissue in the Same 4 Drops of Medium

No.	Area of small explant mm.²	Relative increase	Ratio of areas of explants	Area of large explant mm.²	Relative increase	Difference in relative increase
1.	0.22	10.10	0.65	0.34	11.20	−1.10
2.	.39	21.18	.53	.73	13.85	7.33
3.	1.08	5.18	.87	1.24	5.25	− .07
4.	.84	13.35	.80	1.05	12.52	2.83
5.	.18	50.50	.38	.47	19.29	31.21
6.	.53	11.63	.60	.88	9.46	2.17
7.	.84	11.54	.70	1.20	9.46	2.08
8.	.20	17.20	.72	.28	21.23	−4.03
9.	.44	13.62	.66	.67	10.22	3.40
10.	.24	22.40	.59	.41	19.02	3.38
11.	.25	23.42	.33	.75	12.04	11.38
12.	.39	11.82	.71	.55	10.32	1.50
13.	.70	16.66	.63	1.11	10.25	6.41
14.	.28	20.64	.34	.83	8.08	12.56
15.	.53	17.68	.69	.77	12.05	5.63
16.	.25	39.80	.48	.52	21.63	18.17
17.	.38	27.21	.78	.49	25.04	2.17
18.	.33	19.25	.72	.46	13.95	5.30
19.	.38	16.20	.88	.43	20.30	−4.10
20.	.89	11.55	.89	1.00	9.38	2.17
Mean	0.467±0.040	19.047±1.583	0.648±0.025	0.709±0.044	13.727±0.802	+5.420±1.220

Table 6

Relative Growth of Two Pairs of Fragments of Tissue; the Members of One Pair Close Together in the Medium, Members of the Other Pair Far Apart

No.	Total area of proximate explants mm.²	Relative increase	Ratio of areas of explants	Total area of distant explants mm.²	Relative increase	Difference in relative increase
1.	0.99	12.00	1.25	0.79	16.14	4.14
2.	1.62	10.53	.86	1.89	13.78	3.25
3.	.78	14.20	1.20	.65	27.90	13.70
4.	1.61	6.13	1.14	1.41	10.27	4.14
5.	1.66	10.04	.81	2.04	10.30	.26
6.	.62	11.72	.87	.71	13.43	1.71
7.	.34	27.04	.71	.48	19.53	- 7.51
8.	.73	11.58	.66	1.11	11.56	- .02
9.	.71	19.54	1.09	.65	20.25	.71
10.	1.54	8.78	1.15	1.34	12.10	3.32
11.	0.52	8.39	.52	1.00	14.90	6.51
12.	.85	14.08	.90	.94	10.80	- 3.28
13.	1.13	10.33	.62	1.81	12.76	2.43
14.	1.12	9.18	.59	1.89	10.40	1.22
15.	.50	17.60	.62	.81	18.35	.75
16.	.61	8.84	.53	1.16	10.72	1.88
17.	.46	26.64	.35	1.30	14.33	-12.31
18.	.50	23.40	.93	.54	30.28	6.88
19.	.92	21.34	1.19	.77	26.22	4.88
20.	.39	19.79	.45	.87	26.06	6.27
Mean	0.880±0.066	14.558±0.959	0.822±0.042	1.108±0.073	16.504±0.975	+1.947±0.814

more important factor than volume of medium, although if the size is constant, the volume relations may change the growth considerably (table 3).

Since the fragments placed far apart grew only a little better than those placed close together, and since the smaller of two fragments in the same drop of medium grew better than the larger, at least two alternative explanations are possible. *a*) If there is little diffusion of food materials and inhibiting substances elaborated by the cells through the medium, then the separated fragments would be less inhibited

TABLE 7

Summary of results

TABLE NO.	SERIES NO.	RATIO OF AREAS OF EXPLANTS a/b	VOLUME OF MEDIUM (DROPS) a	VOLUME OF MEDIUM (DROPS) b	RELATIVE INCREASE	PER CENT DIFFERENCE	'STUDENT'S' STATISTICAL PROBABILITY
1	1	0.89	4	4	a > b	2.3	0.6618
	2	0.58	2	2	a > b	33.8	0.0160
2	3	0.71	4	4	a > b	29.5	0.0000
	4	0.55	4	4	a > b	40.5	0.0034
	5	0.19	4	4	a > b	82.8	0.0016
	6′	0.76	2–4	2–4	a > b	18.3	0.0776
	6	1.07	2	4	b > a	24.9	0.0088
3	7	1.02	4	12	b > a	38.5	0.0022
4	7′	0.74	4–12	4–12	a > b	31.7	0.0218
5	8′	0.64	Same 4 drops		a > b	28.3	0.0100
6	8	0.82	4	4	b > a	11.9	0.1234
	9	0.56	4	8	a > b	34.8	0.0008

by accumulation of such harmful products than the pieces placed close together. *b*) If there is very free diffusion, then the separated fragments in this respect have but little advantage over the crowded fragments. It is generally found that vital dyes, iodine, and salts diffuse quite rapidly through the plasma clot; there is, however, little or no data on the rate of diffusion through the plasma clot of lipins, carbohydrates, proteoses, peptones, etc., which the cells may require as food, or of harmful products given off by the tissues.

On the other hand, the fact that the smaller of the two pieces of tissue grew better than the larger when both were

present in the same drop about 1 cm. apart, indicates that the explanation of the results does not involve a free diffusion of harmful products or food. More probably, that, due to the slow diffusion, harmful products are accumulated locally about each fragment of tissue, tending to accumulate faster

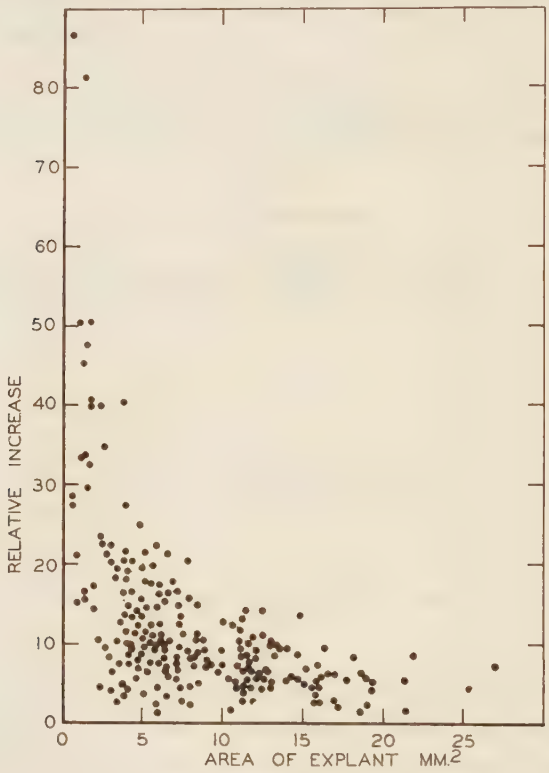

Fig. 2 Area of explant and relative increase. Relative increase is the area of increase in forty-eight hours divided by the area of the explanted fragment of tissue.

about the larger fragment than about the smaller and to inhibit it sooner (table 5). Further, food materials are locally used up sooner by the larger than by the smaller piece of tissue.

The greater the difference in size of original explant, the greater the difference in amount of growth. The greater the

difference in volume of medium the greater the difference in amount of growth. This may be stated diagrammatically: $b > a \geqq d > c$. Thus, all of the evidence from the experiments presented here points to the view that the most favorable conditions for the growth of tissue cells in vitro are relatively large volumes of medium and small fragments of tissue. This condition gives the tissue a large surface / volume ratio, which would appear to be the most favorable for the elimination of inhibiting substances and for the diffusion of food materials, including oxygen into the centrally located cells.

If the data are considered together and plotted, the abcissas representing areas of explants and the ordinates their relative increases, figure 2 is obtained. Here it can be seen that with larger explants there is a corresponding fall in relative increase.

If the data are converted and plotted in another way, the abcissas being the same as in figure 2, namely, the areas of explants, the ordinates, however, being the widths of zones of growth, figure 3 results. Upon fitting a straight line to the data, by the method of least squares, there is obtained the line—

$$Y' = 0.95 + 0.12\ X$$

The coefficient of regression is—

$$b_{yx} = 0.12,$$

with a standard error of—

$$\sigma_{byx} = 0.04.$$

There thus appears to be an increase of width of zone of growth with an increase in size of explant.

Five different cases of the relation between the area of explant, relative increase, and width of zone of growth are possible. These are shown diagrammatically in figure 4, each case representing a somewhat different hypothesis. In each case three sizes of explants are shown by the inner circles. The outer circles represent the appearance of the hypothetical cultures after a definite time period. The computations of area, radii, relative increase (Y), and width of zone of growth

(Y′) can be estimated from the figure. Below the circles the values of the arbitrary examples are plotted for each case.

Case 1 shows a uniform relative or percentage increase. Figure 2 clearly shows this not to be supported by the data. Case 3 is likewise ruled out by figure 2. Cases 2, 4, and 5 are in possible agreement with the data, however, case 4 is at once ruled out by figure 3, leaving cases 2 and 5 for further consideration. Figure 2 and cases 2 and 5 suggest that the amount of growth is proportional to a narrow band of tissue about the edge of the explant. This is supported by the

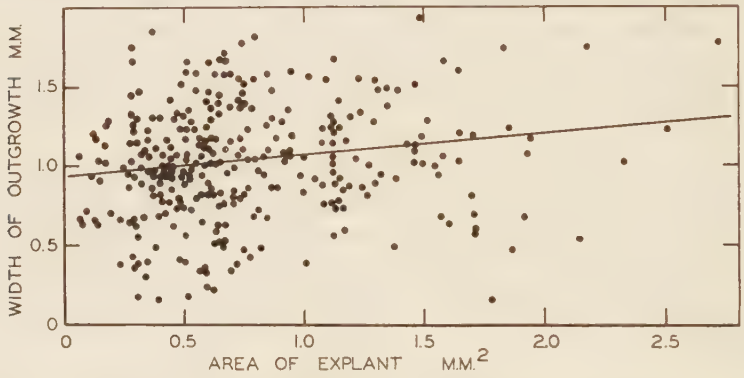

Fig. 3 Area of explant and width of zone of outgrowth. The width of zone of outgrowth was computed from measured areas of cultures. The assumption was made that the areas were circles.

observation that with larger explants, the central part very frequently dies. Since the data of figure 3 shows only a slight increase in width of zone of growth, it is a matter of little import whether the width of zone of outgrowth is considered to be constant or slightly increasing with increase of size of explant. These considerations support the view that the cells in the center of the large explants do not contribute markedly to the growth; the explant does not produce growth proportionately to its size. Only cells in a narrow band on the edge of the explant can get out and grow, hence in the smallest sizes, all the cells may perhaps migrate and divide. Most of the cells of the center of the larger explants may be

either mechanically unable to migrate out or because they are more or less isolated from the source of food and oxygen they may become physiologically inactive or even die. The trend lines of figures 2 and 3 seem to fit case 5 of figure 4 most closely. Such results are contrary to the idea of allelocatalysis, that the presence of greater numbers of cells is correlated with greater relative growth or proportionally increased with that of the zone of growth.

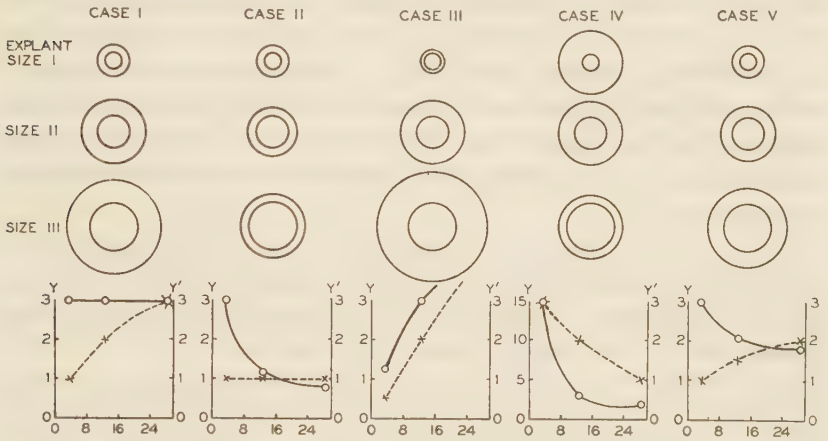

Fig. 4 Diagram of the five possible sets of results that may be expected on the basis of several hypotheses. The inner circles in each case represent areas of explants; there are three sizes for each case. The outer circles represent the areas of cultures after a period of growth. The graphs below show corresponding curves for each case. The smooth line (comparable with fig. 2) with ordinate Y, represents relative increases. The dotted line (comparable with fig. 3) with ordinate Y', shows width of zone of outgrowth. Both abscissas are areas of explants. The arbitrary values used in this construction may be estimated from the diagram.

It may be stated at this point that I have been considering the rapidly dividing cells of tissue culture as ecologically analogous to individual protozoans. Two differences or reservations may be noted: 1) the tissue-culture cells as a population of independent organisms do not move about as rapidly as protozoans and they grow best in a semisolid medium. Both of these factors tend toward stagnation of the medium and limitation in food supply and waste-product removal.

2) If the tissue cells are not kept in rapidly dividing condition, they tend to differentiate. The assumption of tissue cells being independent organisms is supported by the work of Olivo ('30); however, it may still be open to objection on the basis of a number of older papers which claims such cells to be a syncytium.

The effect of varying greatly the volume of medium was shown by Petersen ('29) in the case of Paramecium in which she was unable to show the allelocatalytic effect using two to five drops (0.08 to 0.20 cc.), but showed an acceleration using twenty drops (0.83 cc.). Apparently the smaller volumes in her experiments were more easily exhausted and rendered unfavorable than the larger volumes in which the animals are said to maintain conditions more favorable to themselves.

Darby ('30) found no allelocatalysis with Paramecium grown in an optimum medium. He explains Robertson's allelocatalytic results on the basis of suboptimal hydrogen-ion concentration. It appears that Robertson's—

> medium was away from the optimum pH, and weakly buffered. The organisms tended to change it, and the more he put in at a time, the quicker was the improvement. In the smaller volumes it was easier to bring this about than in the large ones. In too small a volume, however, the accumulation of ammonia would soon retard growth; while in the very large volumes the buffering power of the organisms would be ineffective.

He gives a similar explanation for the experience of yeast growers that larger inocula are more effective in producing growth than smaller inocula, because, unless very carefully prepared, the medium is likely to be off pH.

Again, perhaps the allelocatalytic effect is only shown under adverse conditions, such as the presence of toxins, low oxygen tension, etc., as well as pH deviations. Thus group protection was shown against colloidal silver (Allee and Schuett, '27). A greater number of a group of animals survive in a given volume and concentration of a toxin longer than the same number of animals placed in the same concen-

tration and volume per animal, but isolated, because each individual receives a smaller dose of the toxin, much of the toxin being absorbed on the dead members of the group. No data are available to support this point in tissue culture to my knowledge. Adverse conditions in tissue cultures become reflected in decreased growth and cell death. However, work with extremely small concentrations of toxic substances may yield important information on the point. The work reported here is concerned only with tissues in as favorable a medium as could be obtained.

Another test of the possible benefit of cells to one another, is the capability of cells to survive alone. In an attempt to isolate single cells in an effort to obtain a strain of cells derived from a single source, as is done in the case of bacteria, Fischer ('23) says that:

> If the transplanted piece consisted of a few scattered cells no growth took place, and the cells took on the aspects of degeneration at a time when the control culture (of many cells) seemed to be in perfect condition. By contrast, when the small clumps of cells had been taken from the central portion of the culture where it was dense, growth was extensive. The tissue used in these experiments was derived from a 4 month old strain of fibroblasts. The small fragments were obtained by clean cuts with a sharp cataract knife. After a little practise fragments could be cut so small that they could just be distinguished with the naked eye.

No measurements are given, but again using a somewhat different method, he says: "Cell divisions were not observed in isolated cells under the conditions of the experiment. Growth took place only when the tissue cells were numerous and close to one another." However, he admits the possibility of injury to the cells in the manipulation. Further, Fischer ('23) says:

> When ordinary cultures were cut in two halves and transferred to a fresh medium, it was frequently noted that the cells begin to grow out first from the center of the cut edge of the culture, that is to say from the point at which the cells were most crowded, and only a little later on from the periphery . . . . A culture was permitted to grow for about 20 hours, and then the cover-glass was

unsealed and an incision was made in the periphery of the new growth with an ordinary sharp cataract knife, in order to separate from the mother culture a piece of tissue at the periphery where the cells were rather scattered. The cover-glass was replaced on the slide, it was sealed with paraffin, and an outline drawing was made of the tissue—of the big piece as well as of the small one. The culture was returned to the incubator for another 30 hours; at the end of the second incubation a new drawing was made and the area measured. It was observed that the mother culture had greatly increased in size, but the fragment, separated from it by means of the wound in the clot, consisted of scattered cells and had rather decreased in size.

He concludes by saying that the cells are connected by protoplasmic bridges which are necessary in some way or other for the organic unity and that a piece of tissue grows rather as an organism than as individual cells. In cultures of large mononuclear leucocytes, Carrel and Ebeling ('22) report that if there are too few or too many leucocytes in the culture, they die.

Burrows ('24) found that when oxygen is present in lower concentrations (about one-third atmospheric partial pressure) the cells are stimulated to divide at a greater rate than when it is present in greater concentrations. He explains this by assuming the secretion of various hypothetical substances which at certain concentrations stimulate cell division. When two great a volume of medium is present in proportion to the number of cells, such as occurs when cells are isolated, the stimulating substances supposedly become dilute in the surrounding medium and growth ceases. He suggests that such a difficulty may be overcome by the use of small volumes. He claims that further support to this idea is given by the fact that growing embryonic as well as cancerous tissues are closely packed with but little circulation. Disappearance of the active independent growth of cells in the developing organism may be directly related to the separation of the cells by a rich vascular network and intracellular materials. In tissue cultures, Burrows continues, growth is maximum about the largest fragment into which oxygen may diffuse readily to all of its parts.

C. V. Taylor (personal communication) found that fragments of protozoans and invertebrate eggs from microdissection experiments regenerate better in large volumes of medium (1 cc.) than in hanging drops (0.05 cc.).

Earle and Thompson ('30) studied the influence of the size of the explant upon cultures of fibroblasts of embryonic chicks planted in small and thin hanging drops (0.013 cc.) of plasma and embryonic extract spread over an area 2 cm. in diameter. The range in size of explants used by them varied from only a few cells up to cell clumps of about 1 cu.mm. It was found that the absolute increase in the area generally varied approximately directly as the size of the explant. They found that the cultures of the larger fragments explanted lived longer than the cultures of smaller fragments. In other words, the smaller fragments reached their maximal size sooner than the larger fragments. This is true in my own experience. However, none of the sizes of fragments used in my experiments reached the limit of their growth before forty-eight hours. In the smaller cultures of Earle and Thompson the cells were more uniform in appearance from the center of the explant to the edge, and the cultures reached their maximal size sooner than the larger fragments, although they claim that the food supply was not exhausted, but that the slowing of growth was due to "the influence of some other factor." Some of the intermediate sizes of Earle and Thompson showed more variation and in many cases the final area of some of the smaller sizes was greater than that of some of the larger sizes. This latter is in accord with some of my experiments.

Olivo ('30) isolated six to ten fibroblasts by placing an explant on the moist surface of a new plasma clot, and after one or two hours in the incubator, during which time a few cells migrated from the explant into the plasma clot, he removed the explant and added a drop of plasma. In this way he succeeded in obtaining cultures of fibroblasts. He says that this is evidence for the idea that the cell is an elementary organism capable of life and growth and not necessarily a part of a larger syncytium.

Another worker, Love ('31), studying the occurrence of mitosis in tissue cultures, also found that the rates of increase of pieces of tissue are not independent of size, but that smaller fragments had greater rates of growth than the larger fragments. He postulates: "The variation is almost certainly nutritional in origin. It is obvious that the complete independence of relative rate of increase of area and size of implant would demand an infinite medium."

Although the results presented in this paper do not show the allelocatalytic effect, it is possible that such an effect may be demonstrated by means of tissue culture under other conditions, such as considerably different volumes of medium or sizes of explant, perhaps other types of cells, or under somewhat unfavorable conditions.

SUMMARY

1. In tissue cultures of embryonic chick spindle cells (fibroblast-like cells), the smaller of two fragments within the limits of ordinary tissue cultures (0.1 to 2.5 mm.^{2} in area) has a greater relative increase in area in forty-eight hours. The width of zone of growth is constant or increases slightly with increase in size of explant.

2. If two equally sized fragments of tissue from such cultures are explanted into varying volumes of culture medium (0.02 to 0.12 cc.) as are used in ordinary hanging-drop tissue cultures, the piece in the larger volume has the greater relative increase in forty-eight hours than that in the smaller volume.

3. These results are not in agreement with the idea that cells in tissue culture are mutually beneficial to one another, in that their growth is increased by crowding. On the contrary, it appears that with small explants in large volumes of medium, conditions are most favorable to growth. With larger explants or smaller volumes of medium, the interior cells of the explant are relatively unfavorably situated. This may be because of slow diffusion of nutrients and metabolic products. Thus the amount of growth is a function of the

length of the edge and diameter of the explant, other things being optimum.

I wish to express my sincere thanks to Dr. W. C. Allee, at whose suggestion the problem was undertaken, and to Dr. Wm. Bloom and Dr. S. Wright for their helpful advice during the course of this work.

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